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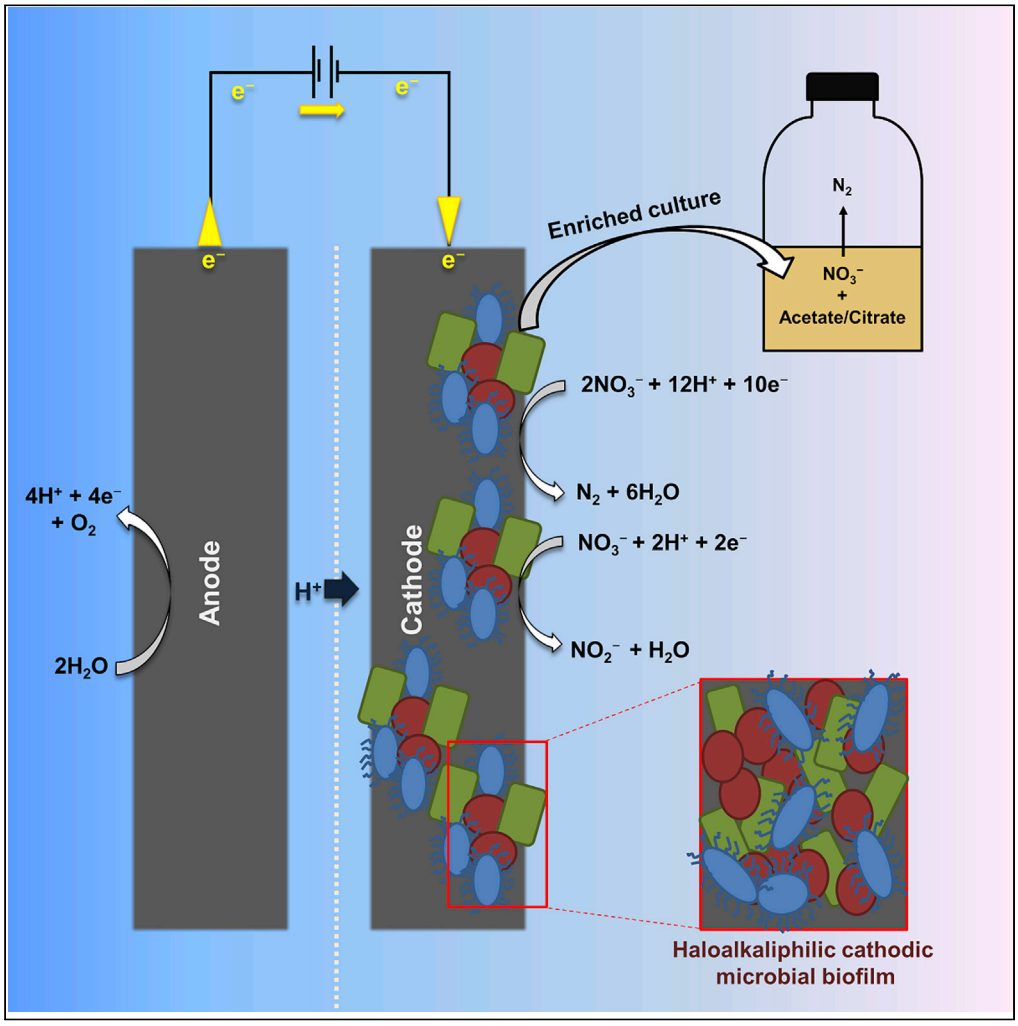

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Highlights

Enrichment of haloalkaliphilic nitrate-reducing microbial biofilm at the cathode

Cathodic reduction current corresponded to the nitrate reduction process

Pseudomonas, *Natronococcus*, and *Pseudoalteromonas spp.* enriched in the cathodic biofilm

Enriched culture reduced nitrate efficiently with soluble electron donor sources

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Electrochemical enrichment of haloalkaliphilic nitrate-reducing microbial biofilm at the cathode of bioelectrochemical systems

Srishti Chaudhary,[1] Ramandeep Singh,[1] Sukrampal Yadav,[1] and Sunil A. Patil[1,2,*]

SUMMARY

Electrotrophic microorganisms have not been well studied in extreme environments. Here, we report on the nitrate-reducing cathodic microbial biofilm from a haloalkaline environment. The biofilm enriched *via* electrochemical approach under 9.5 pH and 20 g NaCl/L salinity conditions achieved $-43.5 \pm 7.2\ \mu A/cm^2$ current density and 49.5 ± 13.2 % nitrate reduction efficiency via partial and complete denitrification. Voltammetric characterization of the biocathodes revealed a redox center with -0.294 ± 0.003 V (*vs.* Ag/AgCl) formal potential putatively involved in the electron uptake process. The lack of soluble redox mediators and hydrogen-driven nitrate reduction suggests direct-contact cathodic electron uptake by the nitrate-reducing microorganisms in the enriched biofilm. 16S-rRNA amplicon sequencing of the cathodic biofilm revealed the presence of unreported *Pseudomonas, Natronococcus,* and *Pseudoalteromonas* spp. at 31.45%, 11.82%, and 9.69% relative sequence abundances, respectively. The enriched nitrate-reducing microorganisms also reduced nitrate efficiently using soluble electron donors found in the lake sediments, thereby suggesting their role in N-cycling in such environments.

INTRODUCTION

Electromicrobiology deals with the study of electrochemical interactions or extracellular electron transfer (EET) processes between microorganisms and solid-state electron donors or acceptors required to maintain respiratory activities and their implications in different environments (Lovley, 2012; Nealson and Rowe, 2016). EET by a microbial cell or biofilm can be either inward or outward transfer of electrons from or to a solid-state electron donor or acceptor. Microorganisms possessing EET capabilities are termed electroactive microorganisms (Nealson, 2017; Logan et al., 2019; Kiran and Patil, 2019). Bioelectrochemical systems (BESs) are used to explore different applications of EAMs and study their EET mechanisms. EAMs are categorized into two main types, namely exoelectrogens and electrotrophs. Exoelectrogens are capable of outward EET, that is, from cells to the solid-state terminal electron acceptors such as mineral oxides and electrodes to achieve respiration, whereas electrotrophs are capable of inward EET, that is, from the reduced minerals or electrodes to the cells to acquire energy (Lovley, 2012; Chiranjeevi and Patil, 2020). Electrotrophs use the acquired electrons to grow and reduce substrates such as carbon dioxide, nitrate, sulfate, and heavy metals.

The components involved in both direct and indirect EET processes have been studied and reported mainly for exoelectrogens but rarely for electrotrophs (Rosenbaum et al., 2011; Shi et al., 2016; Liu and Li, 2020). For instance, *Shewanella oneidensis* MR-1 has been proposed to follow the reverse Mtr pathway of electron transfer for reductive metabolism (Ross et al., 2011) and electron uptake from the cathode electrode (Rowe et al., 2018). Several studies have reported on the electrotrophic mixed-culture microbial biofilms, grown with electron acceptors such as oxygen (Rabaey et al., 2008), H^+ ions (Aulenta et al., 2008), CO_2 (Aryal et al., 2017), heavy metals, polyaromatic hydrocarbons, dyes, and so on (Gregory and Lovley, 2005; Tandukar et al., 2009; Sharma et al., 2020; Fang et al., 2016). Notably, EAMs have been studied mostly from nonextreme environments, and only a few studies have reported the diversity of these microbes inhabiting extreme habitats (Koch and Harnisch, 2016; Rowe et al., 2017; Logan et al., 2019; Yadav and Patil, 2020). In particular, electrotrophs from extreme habitats have been barely studied, limiting our understanding of EET-capable microbes and their role in element cycling under specific environmental conditions and their

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use for various BES applications. Moreover, electrotrophy is a unique energy conservation mode followed by some microbes in the soluble-electron-donor-depleted environments (Logan et al., 2019). It calls for extensive research on microbial resource mining to understand the diversity and EET mechanisms of electrotrophic microorganisms from extreme habitats and explore new strategies to use the EET-capable extremophiles for niche-specific biotechnological applications (Koch and Harnisch, 2016; Schröder and Harnisch, 2017).

Nitrate-reducing microorganisms play a vital role in nitrogen cycling in different environments and can be used to bioremediate nitrate contaminated waters or environments. They are crucial for maintaining and removing the available fixed "N" in nitrate into N_2 via intermediates such as nitrite in natural environments (Jenkins and Kemp, 1984; Codispoti and Christensen, 1985). Both mixed and pure microbial cultures capable of drawing electrons from the cathode have been reported at pH 7 condition (Table 1). Exploring extreme haloalkaline habitats for nitrate-reducing microorganisms capable of electrotrophy could lead to identifying novel microorganisms and electron transfer mechanisms besides understating their role in N-cycling in the soluble-electron-donor-depleted environments. Thus, the present study aimed to enrich and investigate nitrate-reducing microorganisms at the cathode of BES from a haloalkaline environment of the Lonar Lake (located in Buldhana District, Maharashtra, India). The Lonar crater lake is a hypersaline soda lake rich in various nutrients and supports a wide diversity of haloalkaliphilic microorganisms (Paul et al., 2016; Antony et al., 2013; Chakraborty et al., 2020). We first enriched the haloalkaliphilic nitrate-reducing microbial biofilm at the cathode of BES by using an electrochemical cultivation approach. We then tested the enriched biofilm for its nitrate-reducing capability with soluble electron donor sources, selected to match those found in the lake sediments. It was followed by a detailed characterization of the enriched microbial biofilm via electrochemical, microscopic, and 16S-rRNA amplicon sequencing-based analyses. Based on the obtained results, we discuss the electrotrophic nature of the cathodic biofilm and the environmental importance of the haloalkaliphilic microbial groups present in the enriched biofilm.

RESULTS AND DISCUSSION

Chemical analysis of the sediment samples

The pH and salinity of the sediment samples collected from three different sampling locations were 9.5 ± 0.2 and 14.3 ± 1.0 g/L, respectively (Table S1). The salinity of the lake has been reported to vary in a range from 5 to 24 g/L owing to sampling and seasonal variations (Sengupta and Bhandari, 1997; Borul, 2012; Jadhav and Mali, 2018). Based on these observations, the growth medium with a pH of 9.5 and salinity of 20 g/L was used to enrich nitrate-reducing microorganisms. Notably, the sediment samples were found to be rich in various soluble ions such as SO_4^{2-} and NO_3^- (Table S1), which can act as soluble electron acceptors in microbial respiratory processes. In particular, NO_3^- at about 222.4 ± 7.0 mg/L suggests the likely presence of nitrate-reducing microorganisms in this extreme habitat.

Enrichment of the haloalkaliphilic nitrate-reducing microbial biofilm at the cathode

The bioelectrocatalytic reduction current and nitrate concentration in the bulk phase were monitored to check the growth of nitrate-reducing microorganisms at the polarized cathode surface (Figure 1). In the case of enrichment reactor R1, the reduction current started to increase after approximately 40 days (Figure 1A), suggesting a long start-up period, most likely owing to the time required by microorganisms to acclimatize to the challenging solid-state electron donor conditions. In subsequent batch cycles, the increase in the reduction current response was observed, which was accompanied by the decrease in the nitrate concentration. The abiotic-connected control showed no considerable reduction current response and no change in the nitrate concentration. Neither microbial growth nor change in nitrate concentration was observed in the biotic-unconnected control reactor (Figure S1). These observations suggested the occurrence of microbial nitrate reduction process by utilizing cathodic electrons, i.e., the enrichment of nitrate-reducing microorganisms at the cathode surface. On completing the first batch cycle, the enriched biofilm in R1 was used as the microbial inoculum for inoculating reactors R2 (Figure 1B) and R3 (Figure 1C). The scrapping of some biomass from the cathode of R1 decreased the current density considerably in the second cycle, which, however, regained in the third cycle (Figure 1A). The enriched nitrate-reducing biofilm in R1 achieved a maximum current density of $-48.86\ \mu A/cm^2$ with a corresponding nitrate removal efficiency of 57.8%.

Similar bioelectrocatalytic performance in the first batch cycle of R2 was observed but within 15 days (Figure 1B). The use of already enriched biofilm as an inoculum source resulted in a decrease in the start-up

Table 1. A comparative overview of the nitrate-reducing biofilms or microorganisms reported in bioelectrochemical systems

S. No.	Source of microorganisms	Major experimental conditions	Applied potential (V *vs.* Ag/AgCl) OR mode of operation	Maximum current density/ Voltage	Carbon source	Reduced product	Nitrate removal efficiency (%) or rate	Formal potential (V *vs.* Ag/AgCl) of the redox peaks during substrate turnover conditions	Reference
1	*Geobacter metallireducens*	pH 6.8, 30°C	−0.5 V	NR	$NaHCO_3$	NO_2^-	90%	NR	Gregory et al., 2004
2	*Thiobacillus denitrificans*	pH 7, 30°C	−0.606 V	−4 to −5 μA/cm^2	CH_3COOH (Possible electron donor)	NR	NR	NR	Kato et al., 2012
3	*Pseudomonas alcaliphila*	pH 7, 30°C	−0.5 V	−48.75 ± 1.25 μA/cm^2	$Na_3C_6H_5O_7$	NR	72.40 ± 2.09%	NR	Su et al., 2012
4	*Thiobacillus denitrificans*	pH 7, 30°C	−0.705 V	−3.28 μA/cm^2	NO_3	NR	75.62 ± 5.97%	−0.515 V	Yu et al., 2015
5	*Thioclava electrotropha* ElOx9	pH 6.5, 30°C 342 mM NaCl	−0.483 V	−2.45 ± 1.3 μA/cm^2	$NaHCO_3$	NO_2^-	NR	−0.277 ± 0.005 V	Karbelkar et al., 2019
6	Mixed culture enriched from anaerobic sludge dominated by α-proteobacteria, β-proteobacteria, γ-proteobacteria and flavobacteria.	pH 7, 30°C	Fixed current (200 mA)	NR	$NaHCO_3$	N_2 via NO_2^-	NR	NR	Park et al., 2006
7	Mixed community enriched from wastewater sludge dominated by Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Firmicutes, and uncultured bacteria.	pH 7, 22°C 0.05% NaCl	OCV	−18.80 ± 1.6 A/m^3 0.101 ± 0.009 V	$NaHCO_3$	NR	35.21 ± 7.41%	NR	Chen et al., 2010

(*Continued on next page*)

Table 1. *Continued*

S. No.	Source of microorganisms	Major experimental conditions	Applied potential (V *vs.* Ag/AgCl) OR mode of operation	Maximum current density/ Voltage	Carbon source	Reduced product	Nitrate removal efficiency (%) or rate	Formal potential (V *vs.* Ag/AgCl) of the redox peaks during substrate turnover conditions	Reference
8	Mixed community enriched from sludge	pH 7, 30°C	0.7 V by the DC power supply	138.39 μA/cm^2	$NaHCO_3$	NR	91%	−0.2 V	Kondaveeti and Min, 2013
9	Mixed community enriched from anaerobic sludge	pH 7.2, 30°C	Fixed current of 200 mA	NR	$NaHCO_3$	NR	99%	NR	Tong et al., 2013
10	Mixed community enriched from return sludge dominated by Proteobacteria	pH 7.4, 30°C	0.7 V applied by the DC power supply	NR	$NaHCO_3$	NR	88%	−0.13 V	Kondaveeti et al., 2014
11	Mixed culture enriched from fresh water sediments and denitrifying biomass from sewage treatment plant, dominated by Betaproteobacteria, including Rhodocyclales and Burkholderiales.	pH 7, 30°C	−0.25 V and −0.35 V	−210 μA/cm^2 and −320 μA/cm^2	$NaHCO_3$	NR	14-40%	−0.18 V/ −0.24 V and −0.45 V	Gregoire et al., 2014
12	Mixed culture enriched from cathodic biofilm, dominated by *Thiobacillus* sp.	pH 8, 22°C 0.05% NaCl	−0.32 V	−3.60 to −3.78 μA/cm^2	$NaHCO_3$	NR	34.1–54.1%	−0.30 V and −0.70 V	Pous et al., 2014
13	Mixed culture enriched from activated sludge dominated by *Geobacter* sp.	pH 7, 22°C	−0.508 V	NR	$NaHCO_3$	NR	532 mg N/m^2/day	−0.38 ± 0.034 V/ −0.363 ± 0.033 V	Pous et al., 2016

(*Continued on next page*)

Table 1. *Continued*

S. No.	Source of microorganisms	Major experimental conditions	Applied potential (V *vs.* Ag/AgCl) OR mode of operation	Maximum current density/ Voltage	Carbon source	Reduced product	Nitrate removal efficiency (%) or rate	Formal potential (V *vs.* Ag/AgCl) of the redox peaks during substrate turnover conditions	Reference
14	Mixed Community enriched from anaerobic sludge dominated by *Shinella* sp. and *Alicycliphilus* sp.	pH 7, 25°C	−0.905 V	NR	$NaHCO_3$	NO_2^-, N_2 and NH_3	3.5 mg/L/day	NR	Nguyen et al., 2016a
15	Mixed community enriched from anaerobic sludge dominated by *Thiobacillus* sp. and *Paracoccus* sp.	pH 7, 25°C	−0.905 V	NR	$NaHCO_3$	NR	322.6 mg/m^2/ day	NR	Nguyen et al., 2016b
16	Mixed community enriched from pharmaceutical wastewater	pH 6.5–6.6	OCV	0.253 V (OCV)	CH_3COOH (electron donor)	N_2	83%	NR	Nikhil et al., 2017
17	Mixed community enriched from previous enrichment reactor dominated by *Acholeplasma* and *Azoarcus* genera	pH 7.5, 25°C	Fixed current of 5–100 μA	0.73–0.224 V (OCV)	$NaHCO_3$	NR	17%	NR	Ding et al., 2017
18	Mixed community enriched from Lonar Lake sediments dominated by *Pseudomonas*, *Natronococcus*, and *Pseudoalteromonas* spp.	pH 9.5, 25°C 2% NaCl	−0.3 V	-43.5 ± 7.2 $\mu A/cm^2$	$NaHCO_3$	NO_2^- and N_2	49.5±13.2 %	− 0.724±0.003 V and − 0.29±0.003 V	This study

OCV: open-circuit voltage; NR: not reported.

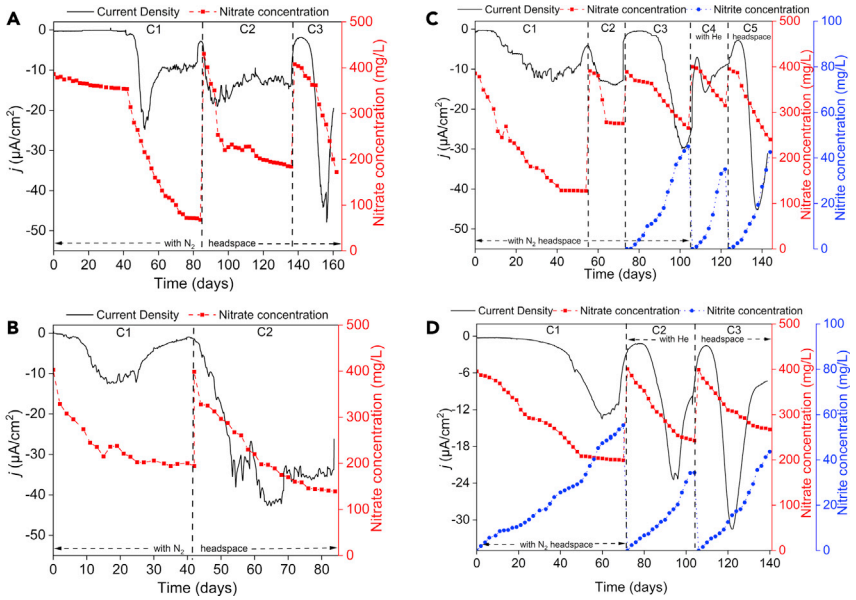

Figure 1. Chronoamperometry and nitrate/nitrite concentration profiles

Bioelectrocatalytic current generation and nitrate reduction by the enriched electrotrophic microbial biofilms in reactors R1 (A), R2 (B), R3 (C), and R4 (D).

time in the current response. A clear relation between the decrease in nitrate concentration and an increase in the reduction current response was observed. The maximum current density and nitrate removal efficiency achieved in this case were $-42.74\ \mu A/cm^2$ and 65.14 % in the second batch cycle, respectively. These data further confirmed the enrichment of microorganisms capable of reducing nitrate by using electrons from the cathode of BESs. In studies conducted at nonextreme pH and salinity conditions, improved nitrate removal efficiencies have been reported (Table 1). For instance, Tong et al. reported 99% efficiency with a mixed microbial culture (Tong et al., 2013). Similarly, Kondaveeti and Min also achieved a maximum nitrate reduction efficiency of 91% with the mixed culture (Kondaveeti and Min, 2013). With a pure culture of *Geobacter metallireducens*, a maximum nitrate removal of 90% has been reported (Gregory et al., 2004).

The third reactor, R3, was run for five cycles; in which, the first three were with N_2 headspace and the rest two were conducted with helium (He) gas in the headspace. In this case, the maximum current density achieved was $-45.05\ \mu A/cm^2$ with a corresponding nitrate removal efficiency of 39.24% along with a production of 42.56 mg/L (2.13×10^{-4} number of moles of $NO_2^- - N$) nitrite and 3.17×10^{-4} number of moles of N_2–N. The corresponding electron recovery for $NO_2^- - N$ and N_2–N was 16.49% and 61.45%, respectively. The nitrate removal efficiencies for the second cycle of R4 and the first cycle of R5 (operated under He gas headspace) were 39.38% and 52.44%, respectively. These values corresponded to 34.36 mg/L (2.18×10^{-4} number of moles of $NO_2^- - N$) and 30.05 mg/L (1.52×10^{-4} number of moles of $NO_2^- - N$) of nitrite formation in R4 and R5, respectively (Figures 1D and S2). In the same cycles, a maximum electron recovery of 74.61% in N_2–N (3.02×10^{-4} number of moles of N_2–N) and 55.83% (6.10×10^{-4} number of moles of N_2–N) was achieved in R4 and R5, respectively.

All the reactors were operated for at least two batch cycles by replenishing the spent medium with a fresh medium. Hence, the instant reduction current response in the subsequent cycles suggested that the bioelectrocatalytic activity (i.e., electron uptake from the cathode) was primarily because of the cathodic biofilm rather than microorganisms present in the bulk phase. The protein estimation *via* Bradford assay (~0.007 mg/L protein concentration) and OD_{600} revealed very little cell growth in the suspension. The protein content of the cathodic biofilm was estimated to be ~0.8 mg/L. These data suggest that microorganisms in the biofilm contributed predominantly to the nitrate reduction process. No significant fluctuation in the pH (9.5 ± 0.29) of the medium was observed throughout the experiments. Moreover, ammonium ion concentration remained almost constant in each batch cycle in the medium, which confirms that no dissimilatory nitrate reduction to ammonium process occurred in the system. These data support that both partial and complete denitrification of nitrate to nitrite and nitrogen occurred in BESs.

The trace amount of H_2 detected in the abiotic setup accounted for about 0.2–0.8% of the total current production of the biotic reactors. Two additional experiments were conducted to check and confirm the role of H_2 in the nitrate reduction process. In the first experiment conducted in the electrically unconnected bioelectrochemical reactor with the enriched cathodic biofilm and fed with H_2 as the only electron source, no decrease in nitrate concentration was observed (Figure S3A). However, about 26% consumption of H_2 was observed after six days of the batch experiment. In the second experiment, serum flasks with H_2 as the only electron source and nitrate as electron acceptor were set and inoculated with the enriched cathodic biofilm. Although OD_{600} increased from the initial 0.035 to a maximum of 0.6, there was no decrease in the nitrate concentration in the serum flasks after four days of incubation (Figures S3B and S3C). The other key observation was about 23.6% consumption of H_2 by the microbes. The results from both experiments suggest that microorganisms that are not involved in the nitrate reduction reaction consumed H_2.

Electrochemical characterization of the haloalkaliphilic nitrate-reducing biofilm

Cyclic voltammograms (CVs) were recorded to detect any redox-active moieties or components in the catholyte and the cathode surface at different conditions before and after the enrichment experiments. The representative CVs for R1, recorded before and after inoculation and during substrate turnover and nonturnover conditions, are shown in Figure 2.

The CV recorded during the substrate turnover conditions (i.e., in the presence of nitrate) showed a typical electrocatalytic curve (Figure 2A, red line trace) and revealed the presence of two redox-active moieties with midpoint potentials of -0.724 ± 0.003 *V* and -0.294 ± 0.003 *V vs.* Ag/AgCl (Figure 2B). Redox-active moieties with similar formal potential were also observed in the CV recorded under the substrate nonturnover conditions (i.e., absence of nitrate) (Figure 2A, black dashed line trace). Based on the cathodic potential (-0.3 *V*) that was applied for enriching the biofilm, it can be deduced that the redox-active moiety with a formal potential of -0.294 ± 0.003 *V* might be involved in the microbial electron uptake process from the cathode for nitrate reduction. Attributing any role to the other moiety, with a low formal potential of -0.724 ± 0.003 *V*, that is not involved in the actual microbial electron uptake process during CA experiments would be speculative. However, based on the literature on nitrate-reducing microorganisms in BESs, this particular redox component might be involved in the nitrite reduction process (Pous et al., 2014). Similar CV behavior was also observed in the case of nitrate-reducing biocathodes of R2, R3, and R4 (Figure S4- I, II, and III). Such redox peaks have been reported previously for the nitrate-reducing biocathodes. For instance, Gregoire et al. and Kondaveeti and Min reported redox peaks with a formal potential of -0.18 V and -0.2 V at pH 7 (which are equal to -0.328 V and -0.348 V at pH 9.5), respectively (Gregoire et al., 2014; Kondaveeti and Min, 2013). These are close to the redox peak with a formal potential of -0.294 ± 0.003 *V* found in the case of the haloalkaliphilic biofilm in this study. Pous et al. reported a redox-active moiety at -0.7 V at pH 8 (equals to -0.789 V at pH 9.5) (Pous et al., 2014). It is close to the second redox-active moiety observed at -0.724 ± 0.003 *V* in our study. Some other redox peaks have also been reported for nitrate-reducing biofilms, as summarized in Table 1.

The CVs recorded with the fresh cathodes in the filtered spent medium of all reactors showed no redox peaks, thereby suggesting the absence of any soluble electron mediators or components in the medium (Figures 2C and S4C- I, II and III). It also suggests that the nitrate-reducing biofilm enriched at the cathode surface most likely follows the direct mode for electron uptake and suggests its electrotrophic nature. A redox peak with a midpoint potential of approximately -0.637 ± 0.004 *V* was observed in all the control CVs, that is, recorded before and after inoculation. It was most likely owing to the presence of a redox-active component in the medium or at the electrode surface. Hence, it is not considered as the redox-active component of the microbial biofilm grown or enriched at the cathode surface.

Nitrate reduction by the enriched cathodic microbial biofilm with soluble electron donor sources

The ability of the cathodic biofilm to grow by using the soluble electron sources was tested using citrate and acetate that are present in the lake sediments. Two sets of experiments were conducted with either He or N_2 in the serum flask headspace for each electron donor condition. An increase in the turbidity (i.e., OD_{600}) corresponding with nitrate reduction suggested the growth of haloalkaliphilic nitrate-reducing microorganisms with the soluble electron donor (Figures 3A, 3B, S5A, and S5B). About $45.92 \pm 9.73\%$ of nitrate reduction and $85.93 \pm 0.29\%$ of citrate oxidation along with 6.35×10^{-5} moles of N_2 production

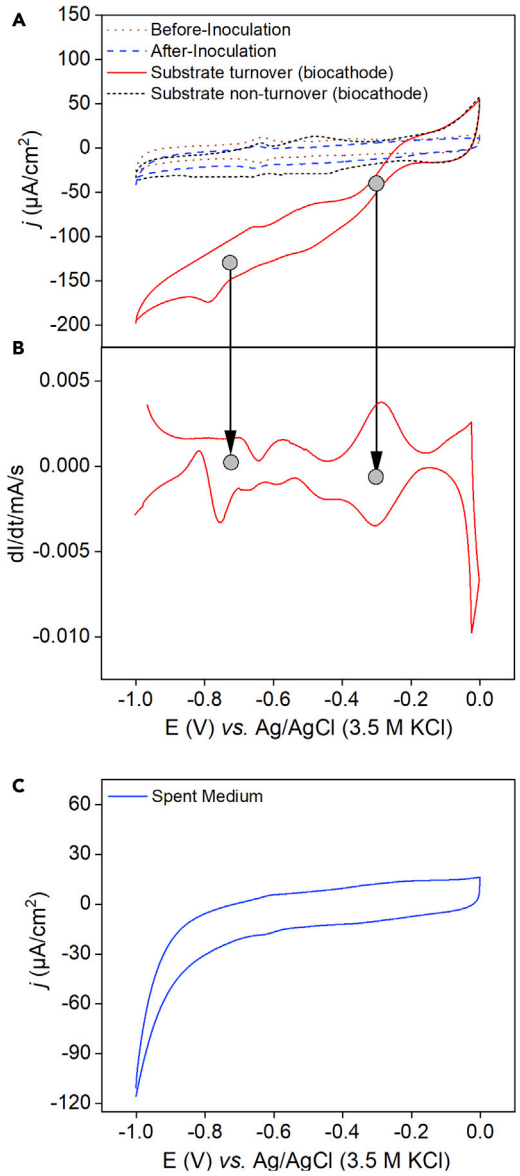

Figure 2. Representative cyclic voltammograms recorded under different conditions for R1

(A) CVs at different conditions, (B) first derivative of the CV recorded under the substrate turnover condition, and (C) CV of the fresh electrode in a filtered spent medium of R1.

were observed at the end of the third batch cycle in He-sparged serum flasks. With acetate as the electron donor, the observed microbial growth in terms of OD_{600} was lesser than citrate. About 75.59 $\pm$ 1.44% acetate oxidation and 93.12 $\pm$ 0.48% nitrate reduction along with 9.87 $\times$ 10^{-5} moles of N_2 production were observed at the end of the third cycle in He-sparged serum flasks (Figures 3A and 3B). Nitrite concentrations increased for the first two days of each batch cycle and then subsequently decreased to zero at the end of the cycles. Similar growth and nitrate reduction trends were observed in the N_2-sparged serum flasks (Figures S5A and S5B). Neither increase in OD_{600} nor decrease in nitrate concentrations was observed in the biotic and abiotic controls (Figures 3C, 3D, S5C, and S5D). These observations suggest that no nitrate reduction occurred under the abiotic condition and in the absence of an electron donor source for microbial cells in the biotic control. The OD data reveal a much shorter lag phase (about 24 h) than the growth under insoluble electron donor (i.e., cathode) and autotrophic conditions in electrochemical cultivation experiments. It suggests the slow growth of nitrate-reducing microorganisms under the electroautotrophic

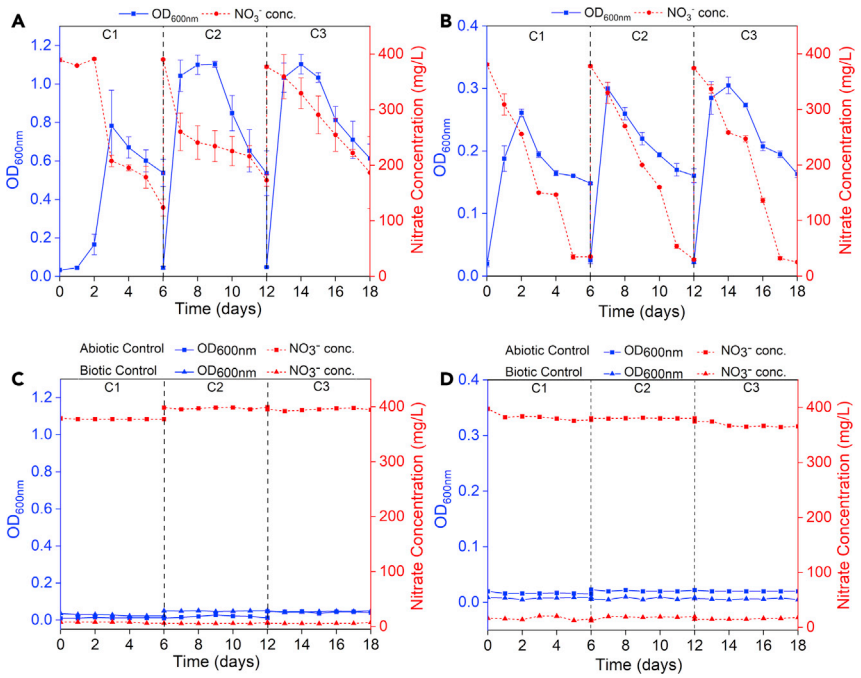

Figure 3. Nitrate reduction profiles of the enriched culture with different electron donors

Microbial growth (in terms of OD_{600}) and nitrate reduction profiles of the enriched nitrate-reducing culture over three batch cycles of the serum flask experiments (n = 3; with He gas in the headspace) with citrate (A) and acetate (B) as electron donors. The data of respective abiotic and biotic control experiments are presented in (C) and (D).

condition. The serum flask experiment observations suggest the role of enriched microorganisms in the cycling of both carbon and nitrogen elements under the haloalkaline growth conditions.

Visualization of the microorganisms at the cathode surface and in the bulk phase of reactors

The scanning electron microscopy (SEM) imaging revealed the presence of microbial cells at the cathode surface (Figures 4A and 4B) and in the bulk phase (Figures 4C and 4D) of bioelectrochemical reactors. In addition, in the suspension of the serum flasks, oval-shaped cells were observed (Figures 4E and 4F). These results confirm the growth of microorganisms in the experiments conducted under both insoluble and soluble electron donor conditions. The biofilm formation at the cathode surface was, however, not uniform and instead was in patches (Figure 4A). Nevertheless, the microbial growth at the cathode surfaces, along with the electrochemical data, confirms the enrichment of haloalkaliphilic nitrate-reducing biofilm. The weight percentage data of FEG-SEM-EDS revealed the absence of any large elements on the surface of biocathodes and abiotic cathode except for the presence of elements, mainly C and O (at 29.17 wt.% and 8.16 wt.%, respectively) and trace amounts of elements such as Al and Ir (at 0.63 wt.% and 0.35 wt.%, respectively) (Figure S6). These might be involved in the catalysis of H_2 evolution at the cathodic surface.

Microbial community composition of the enriched nitrate-reducing electrotrophic biofilm

The 16S-rRNA-amplicon-sequencing-based analysis of the enriched cathodic biofilm revealed the presence of species belonging to *Pseudomonas,* followed by *Natronococcus,* and *Pseudoalteromonas* at 31.45%, 11.82%, and 9.69% relative sequence abundances, respectively (Figure 5). In the Lonar lake sediments, that is, the inoculum source, *Pseudomonas*, *Natronococcus,* and *Pseudoalteromonas* genera were found to be at only 0.264%, 1.815%, and 0.762% relative sequence abundances, respectively (Figure 5). These data suggest that the enriched microbial biofilm through the electrochemical cultivation approach led to selecting a few groups at the cathode capable of growing on the insoluble electron donor source. The most abundant genera present in the cathodic biofilm have been reported for their nitrate-reducing metabolic capabilities in different experimental conditions (Tindall et al., 1984; Mulla et al., 2018; Su et al., 2012). In particular, microorganisms belonging to the *Pseudomonas* genus are known for their

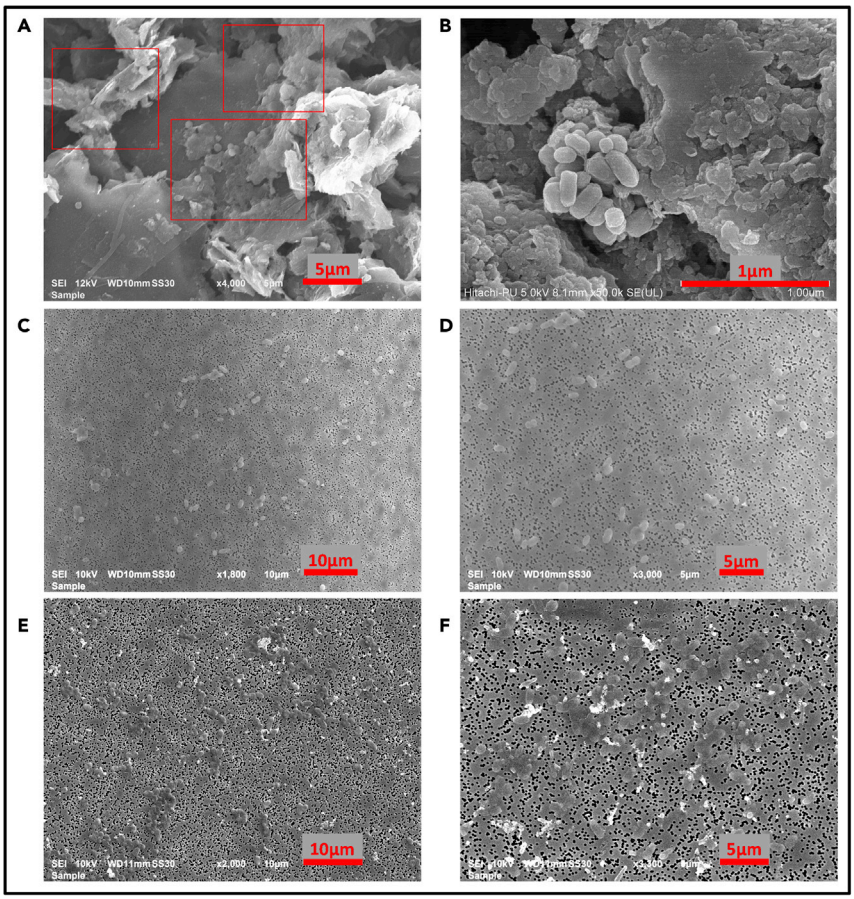

Figure 4. Visualisation of the microorganisms *via* scanning electron microscopy

Representative SEM images for the enriched nitrate-reducing microorganisms at the cathode surface (A and B) and in the bulk phase (C and D) of BESs. The images in panels (E) and (F) are for the nitrate-reducing microbial cells grown with citrate as a soluble electron donor in serum flasks.

electroactivity (Logan et al., 2019). For instance, the pure cultures of *Pseudomonas, namely, Pseudomonas alcaliphila* (Su et al., 2012) and *Pseudomonas aeruginosa* (Jia et al., 2017) have been reported to reduce nitrate at pH 7 (Table 1). As far as the extreme habitats are concerned, this is the first study that reports on the haloalkaliphilic *Pseudomonas* spp. that most likely possesses the nitrate-reducing capability.

A few *Natronococcus* species have been reported for nitrate-reducing capability but not for electroactivity or electrotrophic properties. For instance, haloalkaliphilic *Natronococcus occultus* and *Natronococcus amylolyticus* have been reported to grow optimally at 9.5 pH and 20%–22% salinity by reducing nitrate to nitrite using glucose, ribose, or sucrose as the carbon and electron source (Tindall et al., 1984; Kanai et al., 1995). Both species have been isolated from the East African soda lake, Lake Magadi, *via* conventional cultivation approaches. Another species named *Natronococcus roseus*, isolated from Chagannor Lake, China, has also been reported to reduce nitrate (Corral et al., 2013). The presence of *Natronococcus* in the enriched cathodic biofilm in our study suggests that this genus might possess the traits that facilitate their growth under electrotrophic conditions.

Similarly, several *Pseudoalteromonas* spp., such as *Pseudoalteromonas arabiensis, Pseudoalteromonas lipolytica,* and *Pseudoalteromonas prydzensis,* isolated from the oxygen-depleted regions of Arabian Sea, have been reported to be involved in nitrate and nitrite reduction processes (Mulla et al., 2018). They also possess various genes involved in the nitrogen cycle (Cai and Jiao, 2008). However, they have not been reported for nitrate reduction process using the insoluble electron donors such as polarized cathodes. Hence, the enrichment of *Pseudoalteromonas* in the cathodic biofilm in this study suggests its

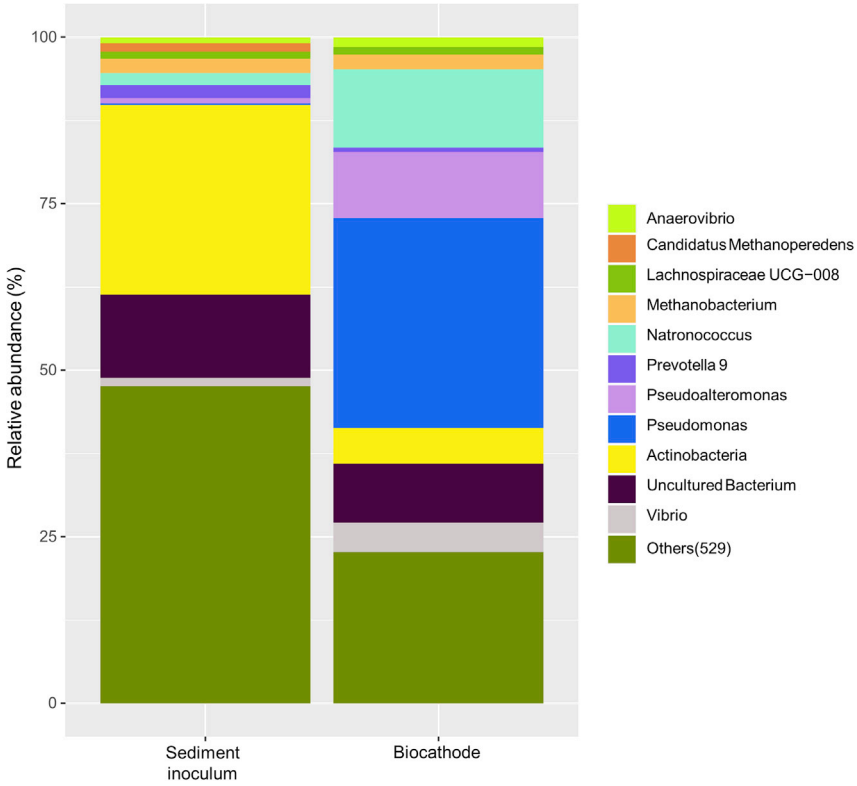

Figure 5. 16S rRNA-amplicon-sequencing-based microbial community composition in the Lonar lake sediments used as the inoculum source (sediment inoculum) and the enriched biofilm at the cathode surface (biocathode)
Others represent the microbial communities present at < 1% relative sequence abundance.

probable electrotrophic nature. *Actinobacteria* and *Vibrio,* with a relative sequence abundance of 5.4% and 4.4%, respectively, have been reported to act as nitrate reducers in acidic or neutral and estuarine environments (Palmer and Horn, 2015; Zenova et al., 2011; Macfarlane and Herbert, 1982). Thus, these can also be implicated in nitrate reduction in BESs. Some other microbial communities, namely, *Geoalkalibacter, Halalkalicoccus,* and *Azoarcus* that are known to reduce nitrate, were also present in the enriched electrotrophic EABs, but at very low relative sequence abundances of <0.00004%. Among *Halalkalicoccus* genus, *Halalkalicoccus tibetensis* (haloalkaliphile) and *Halalkalicoccus paucihalophilus* (halophile) isolated from the Lake Zabuye and Lop Nur region, China, respectively, are capable of reducing nitrate to nitrite. Finally, *Azoarcus* species strain PA01^T has also been reported to use nitrate as a terminal electron acceptor (Junghare et al., 2015). *Methanobacterium, Anaerovibrio,* and *Lachnospiraceae* UGC-008 with relative sequence abundances of 2.2%, 1.4%, and 1.2%, respectively, were found in the enriched biofilm. However, their role has not been reported for the nitrate reduction process, so they might be involved in syntrophic relationships in the mixed microbial biofilm for sharing electron donors or acceptors. For instance, the traces of electrochemically produced H_2 in BESs were not used by the nitrate-reducing microorganisms but most likely supported the growth of methanogens in the enriched biofilm. In addition to these genera, an uncultured bacterium was found in the cathodic biofilm at a relative sequence abundance of 8.8%. Because its identity is not clear, its role cannot be discussed.

Role of enriched microorganisms in the cathodic electron transfer and nitrate reduction process

Many *Pseudomonas* spp. can produce mediators such as pyocyanin and phenazine-1-carboxamide, which are known to enhance their electron transfer process and thereby current production efficiency of bioelectrochemical systems (Rabaey et al., 2005; Venkataraman et al., 2010). A few studies have reported certain *Pseudomonas* spp. to produce phenazine compounds in either alkaline or saline conditions (Zhang et al., 2011; Patil et al., 2016; Yuan et al., 2020). For instance, Sahoo et al. reported *P. aeruginosa* to produce phenazine compounds at saline and moderately alkaline (pH 8) conditions (Sahoo et al., 2019). The high-resolution mass spectroscopy analysis of

the cathodic biofilm and the spent medium revealed the absence of both pyocyanin and phenazine-1-carboxamide in our study (Figures S7 and S8). These results confirm the CV observations on the lack of any soluble mediators in the bulk phase. These data and the lack of hydrogen-driven nitrate reduction together suggest that the electron transfer was biofilm-associated and most likely *via* direct electron transfer in the case of nitrate-reducing microorganisms. One plausible way of electron transfer among microorganisms in a mixed community is direct interspecies electron transfer (Semenec et al., 2018; Li et al., 2020; Mostafa et al., 2020). It can involve uptake of cathodic electrons by a non-nitrate-reducing microbial group and then their transfer to nitrate-reducing microbes, that is, syntrophic exchange of electrons among different microbial groups to fulfill their growth or metabolic requirements. However, this possibility needs to be confirmed through dedicated experiments with the pure cultures of microbes present in high relative sequence abundance in the nitrate-reducing cathodic biofilm.

The Lonar lake sediments contain nitrate at a considerable concentration (222.4 mg/L). In addition, organic and inorganic electron donor sources such as acetate, citrate, Fe, and Mn are present in the lake sediments. Although present at the low relative sequence abundance in sediments, the capability of different microbial groups such as *Pseudomonas, Natronococcus* and, *Pseudoalteromonas* present in the enriched culture to reduce nitrate to nitrite and N_2 *via* partial or complete denitrification processes using soluble electron donor sources suggests their possible role in N cycling in the haloalkaline environment. The nitrate reduction by the enriched culture with cathode as the insoluble electron donor also infers its probable capability to use reduced Fe minerals as the source of electrons in the sediments (Beller et al., 2013; Yu et al., 2015; Liu et al., 2019). However, the proposed role of enriched microbes in the cycling of N, C, and Fe elements needs to be investigated in dedicated experiments with the pure culture isolates.

To summarize, none of the enriched microbial groups in the cathodic biofilm, with the highest relative sequence abundances observed in this study, were reported earlier for nitrate reduction under highly saline-alkaline conditions. The following observations suggest the electrotrophic nature of the enriched nitrate-reducing microorganisms in the cathodic biofilm.

- Most electric current response was observed only in the test reactors with cathodic microbial biofilm and not in the control reactors.
- Soluble redox mediators, in particular, the most commonly produced ones by *Pseudomonas* spp., namely pyocyanin and phenazine-1-carboxamide, were not detected in the reactors.
- No artificial redox mediator was supplied in the medium.
- The trace amount of electrochemically produced H_2 in the abiotic experimental setup contributed only to 0.2%–0.8% of the total current density produced in the biotic reactors. Moreover, it was not used as the source of electrons for nitrate reduction by the enriched microorganisms in the cathodic biofilm.

Conclusions

The electrochemical cultivation resulted in the enrichment of a haloalkaliphilic nitrate-reducing microbial biofilm composed mainly of *Pseudomonas, Natronococcus,* and *Pseudoalteromonas* spp. at the cathode surface. The nitrate-reducing microorganisms in the enriched biofilm most likely followed the direct-contact electron uptake mechanism rather than mediated by redox shuttles and hydrogen to reduce nitrate under autotrophic conditions. An unknown redox-active moiety putatively involved in the electron uptake process was revealed by electrochemical analysis of the biocathodes. The enriched nitrate-reducing culture also grew faster by using soluble electron donor sources under heterotrophic conditions. By reporting on the haloalkaliphilic nitrate-reducing biofilm composed of several microbial groups under electroautotrophic conditions, this study expands the known habitats for both electrotrophs and nitrate reducers. Further work on the isolation and detailed characterization of dominant microorganisms for electroactivity and nitrate reduction is expected to broaden the diversity of both extreme electroactive and nitrate-reducing microorganisms.

Limitations of study

This study reports on the haloalkaliphilic nitrate-reducing microorganisms, which are slow growing under electroautotrophic conditions. The redox center involved in the electron uptake process cannot be

attributed to any specific microbial group or component because of the mixed culture biofilm. In addition, specific electrotrophic microorganisms cannot be pointed out conclusively in the enriched mixed biofilm.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102682.

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AUTHOR CONTRIBUTIONS

S.A.P. conceived and designed the study. S.C. conducted the experiments and analyzed the data. R.S. and S.Y. helped in acquiring and analyzing the data. All the authors wrote and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENTS or RESOURCES	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Nitrogen gas	Sigma gases, India	N/A
Helium gas	Sigma gases, India	N/A
Hydrogen gas	Sigma gases, India	N/A
Glutaraldehyde	Sigma-Aldrich	Cat#G5882
Paraformaldehyde	Sigma-Aldrich	Cat#158127
Critical commercial assays/Kits		
Bradford Assay	Sigma-Aldrich	Cat#2740-1KIT
FastDNA<191> 2 mL SPIN Kit for Soil	DNeasy® PowerSoil® Pro kit Qiagen, Germany	Cat#116560200
Deposited data		
Raw data	This paper	NCBI-SRA: SRR12506991
Oligonucleotides		
V3 specific forward primer 5′-GCCTACGGGNGGCWGCAG-3′	Eurofins Scientific, India	N/A
V4 specific reverse primer 5′-ACTACHVGGGTATCTAATCC-3′	Eurofins Scientific, India	N/A
Software		
EC Lab	BioLogic Science Instruments, France	N/A
OriginPro 2021	OriginLab®	https://www.originlab.com/2021
R Packages for Bar plots	R packages	https://r-pkgs.org/
Other		
Proton Exchange Membrane (PEM) (Nafion 117)	Sigma-Aldrich	Cat#274674-1EA
Graphite Plate 0.5 cm thickness (30 X 30 cm)	Ipgi Instruments, India	N/A
Titanium Electrode coated with mixed metal oxides for water splitting reaction at anode (5 cm X 2.5 cm) with thickness of 1mm connected with 10 cm titanium wire of 1 mm thickness	Ipgi Instruments, India	N/A
Ag/AgCl (3.5 M KCl) electrode	BioLogic Science Instruments, France	Cat#RE-1B
Potentiostat (VMP3)	BioLogic Science Instruments, France	N/A
Fuel Cell Glass Reactors (350 mL)	Jain Scientific Glass Works, India	N/A
Serum Bottle: volume 100 mL, clear glass bottle, O.D. 51.7 mm × H (94.5 mm), pkg of 36 ea	Sigma-Aldrich	Cat#33110-U
UV-Vis spectrophotometer: PhotoLab® 7600	Xylem	https://www.xylemanalytics.com/en/general-product/id-364/spectrophotometer-photolab%C2%AE-7600-uv-vis—wtw
pH meter: Jenway 3510	Jenway®	http://www.jenway.com/product.asp?dsl=285
Gas Chromatograph: GC-TCD, Micro GC, Agilent 490	Agilent Technologies	https://www.agilent.com/en/product/gas-chromatography

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Continued

REAGENTS or RESOURCES	SOURCE	IDENTIFIER
HPLC: Agilent 1260 Infinity II, Hiplex H column	Agilent Technologies	https://www.agilent.com/en/product/liquid-chromatography
Electrospray Ionization High-Resolution Mass Spectrometry (HR-MS-ESI): Waters Synapt G2-Si Q ToF Mass Spectrometer	Waters	https://www.waters.com/waters/en_IN/Mass-Spectrometry/nav.htm?cid=514257&locale=en_IN
JEOL JEC-1600 Auto-Fine Coater	JEOL Ltd., Japan	https://www.jeol.co.jp/en/
Scanning Electron Microscope: JEOL JSM-6010 PLUS-LS	JEOL Ltd. Japan	https://www.jeol.co.jp/en/
FEG-SEM-EDS: Hitachi SU8010 series	Hitachi	https://www.hitachi-hightech.com/global/about/news/2011/nr20110214.html
Nanodrop: Genova Nano – 4359, Jenway, Cole-Parmer, UK	Jenway®	http://www.jenway.com/product.asp?dsl=885
Qubit fluorimeter: V.3.0, Thermo Fischer Scientific, USA	Thermo Fischer Scientific	Cat#Q33238

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sunil A. Patil (sunil@iisermohali.ac.in, sunilmicro12@gmail.com).

Materials availability

No unique reagents were generated in this study.

Data and code availability

All the data generated or analyzed during this study are included in this article and its supplemental information. All the raw sequencing data files are available on the NCBI short-read archive under the SRR12506991 accession number.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

No experimental model and subject was used in this study.

METHOD DETAILS

Experimental conditions

The bioelectrochemical experiments were performed under strictly anaerobic conditions at a temperature of 25±2 °C. All potential values are reported against the Ag/AgCl reference electrode (3.5 M KCl; 0.205 V *vs.* standard hydrogen electrode (SHE)) and at the pH condition of 9.5, if not stated otherwise. Bioelectrochemical current was recorded using the EC Lab software (Biologic), and all the graphs were plotted using OriginPro 2021 software.

Sediment sampling and characterization

Sediment samples from the Lonar Lake (Buldhana district, Maharashtra state, India) were collected in amber-colored sampling containers from up to a depth of 1 ft. and were stored at 4 °C. Different parameters were analyzed following the standard analytical protocols as per "Standard Methods for the Examination of Water and Wastewater" (Rice et al., 2012). These include pH, salinity, conductivity, ammonia, chemical oxygen demand (COD), phosphate, sulfate, and nitrate (Table S1). The suspension prepared from the sediment samples (after removing coarse particles) was used as the microbial inoculum source in bioelectrochemical reactors.

Bioelectrochemical reactor components and medium composition

Two-chambered glass reactors (each chamber with 350 mL empty bed volume) with a three-electrode configuration, separated by a proton exchange membrane (PEM), were used as the bioelectrochemical reactors (Figure S9). The working and counter electrodes were graphite plate and dimensionally stable Ti plate coated mixed metal oxide, respectively, with a projected surface area of 7.0125 cm^2. The reference was an Ag/AgCl (3.5 M KCl, RE-1B, Biologic Science Instruments, France) electrode. The working electrode, which served as the cathode, was pre-treated before use by the acid-alkali method. The PEM (Nafion 117, Sigma-Aldrich) was pre-treated by sequentially heating it at 70-80 °C for 1 hour, each in distilled water, 2% H_2O_2, distilled water, 1 N H_2SO_4, and distilled water.

A modified M9 medium with 20 g/L salinity and 9.5 pH was used as a catholyte or growth medium (200 mL) in the cathode chamber. Its composition is as follows (per L of distilled water): 4.33 g Na_2HPO_4, 2.69 g NaH_2PO_4, 20 g NaCl, 4.3 g Na_2CO_3, 0.13 g KCl, 0.31 g NH_4Cl, 12.5 mL vitamins, and 12.5 mL trace elements (Yadav and Patil, 2020). Sodium nitrate (10 mM) and sodium bicarbonate (10 mM) were used as the terminal electron acceptor (TEA) and carbon source, respectively. The graphite cathode served as the sole electron donor. The catholyte lacked any other soluble or insoluble electron donors and acceptors. The anolyte (pH 7) composition is as follows (per L of distilled water): 4.33 g Na_2HPO_4, 2.69 g NaH_2PO_4, 20 g NaCl, 0.13 g KCl, and 0.31 g NH_4Cl. The catholyte was flushed with either 99.999% inert N_2 or He gas (Sigma Gases, India) at least for 20 min before starting any experiment. All the reactor openings were sealed with butyl rubber stoppers. The experiments with He gas in the headspace of bioelectrochemical reactors were conducted to detect N_2 formation due to the complete denitrification process.

Electrochemical cultivation of the haloalkaliphilic nitrate-reducing electrotrophic biofilms

The electrochemical enrichment experiments were conducted at an applied potential of -0.3 V at the working electrode (i.e., cathode) using a potentiostat (VMP3, BioLogic Science Instruments, France). This potential was chosen to facilitate microbial nitrate reduction *via* a direct electron transfer process. Bioelectrocatalytic current response (i.e., the reduction current response generated as a result of the electron draw from the cathode) was recorded as a function of time using the chronoamperometry (CA) technique (Yee et al., 2020; Chiranjeevi and Patil, 2020). Cyclic voltammetry (CV) was performed in a potential window of -1 to 0 V and at a scan rate of 1 mV/s to check the presence of any redox-active moiety or components in the catholyte medium and at the cathodic surface under different conditions (Harnisch and Freguia, 2012). These include before and immediately after microbial inoculation in medium (i.e., abiotic cathode), after the growth of microbial biofilm at the cathode (i.e., biocathode) during substrate turnover and non-turnover conditions, and in filtered spent medium with a fresh electrode. The electrochemical cultivation experiments were conducted for at least two batch cycles to check the variability and reproducibility in the bioelectrocatalytic performance by the enriched cathodic biofilm. Two control experiments, *viz.* abiotic-connected (without microbial inoculum but at an applied cathode potential) and biotic-unconnected (inoculated with the microbial source but without any applied cathode potential) were performed to confirm the observations of the main experiments. The enriched culture was further tested for electroactivity and nitrate-reduction capability in electrochemical and serum flask experiments. For electrochemical tests, similar experimental conditions and approaches, as described earlier, were used.

Serum flask experiments

The ability of the enriched cathodic biofilm to reduce nitrate using soluble electron donors was tested in serum flasks. The enriched microbial biofilm from the cathode was used as an inoculum in serum flasks hosting 40 mL of hypersaline-alkaline medium with sodium citrate or sodium acetate as the soluble electron donor and nitrate as the terminal electron acceptor. These experiments were conducted either with N_2 or He gas in the headspace. The He condition enabled the detection of N_2 formation as a result of the complete denitrification process. Before inoculation, serum flasks were sparged with N_2 and sealed with butyl rubber and aluminium crimp. After autoclaving, they were inoculated and incubated undisturbed at 25 ± 2°C. These experiments were conducted in triplicates and for three batch cycles. Two control experiments, *namely,* abiotic (without inoculum) and biotic (with inoculum but without nitrate), were also conducted.

Experiments with H_2 as the only electron donor source

The role of H_2 that was produced in very low concentration at the applied cathode potential in the nitrate reduction process by the enriched biofilm (i.e., H_2-driven nitrate reduction) was checked by conducting two experiments. In the first experiment, 10 mL (20% of the headspace) of H_2 was added to the reactor with an

enriched cathodic biofilm and a fresh sterile medium containing 10 mM nitrate. It was electrically disconnected from the potentiostat to check whether nitrate reduction occurs when only H_2 is provided as the electron donor source (and no cathodic electrons) to the enriched microorganisms. In the second experiment, serum flasks were set as explained earlier, but instead of adding a soluble electron donor, H_2 (1.3E-03 moles) was provided as the only electron donor source and inoculated with the enriched cathodic biofilm. In both cases, the concentrations of nitrate and H_2 were monitored. In serum flasks, OD_{600} was also monitored.

Chemical analysis and calculations

The catholyte samples were analyzed for OD_{600}, pH (Jenway 3510 pH meter), and nitrate concentrations regularly at an interval of 48 h and 24 h in bioelectrochemical and serum flask experiments, respectively. Nitrate was analyzed by standard ultraviolet spectrophotometric screening method (Rice et al., 2012) using a UV-Vis spectrophotometer (PhotoLab® 7600). Total nitrate removal efficiency is reported as the percent difference between the initial (at the start of each batch cycle) and final (at the end of each batch cycle) concentrations. Ammonium ions were occasionally analyzed to check the presence of any dissimilatory nitrate reduction by the phenate method (Rice et al., 2012). N_2 gas in the headspace of the reactor was detected at the initial and final stage of each batch cycle by gas chromatography (GC-TCD, Micro GC, Agilent 490). Nitrite concentrations were measured using the Griess Reagent method (Rice et al., 2012). Acetate and citrate concentrations in the serum flask experiments were measured by using HPLC with RI detector (Agilent 1260 Infinity II, Hiplex H column, 5μM H_2SO_4 as mobile phase, flow rate 0.5 ml/min, Temperature 50 C). The presence of soluble redox mediators in the bulk phase and cathodic biofilm of the bioelectrochemical reactors was checked using Electrospray Ionization High-Resolution Mass Spectrometry (HR-MS-ESI) at 2kV electrospray voltage. For this, different dilutions (1, 0.75, 0.5, and 0.25) of filtered suspension samples and biofilm samples were compared with the fresh growth medium. The bioelectrocatalytic current responses are reported as current density by normalizing the achieved current with the cathode projected surface area. The microbial growth in the bioelectrochemical reactors was also analyzed based on protein estimation by Bradford assay.

The electron recovery for $NO_2^- - N$ and N_2 –N was estimated based on the ratio of the product of faraday's constant (F), number of moles of product formed (n), and number of electrons used by each mole (b) to the total charge produced/transferred over time (*It*) in each batch cycle. The formula used for the same is given below.

$$\textit{Electron recovery}\ (\%) = \frac{Fnb}{\sum_0^{t_i} It} * 100$$

Analysis of the nitrate-reducing cathodic microbial biofilms

Scanning electron microscopy (SEM). SEM was used to check microbial growth at the cathode surface and in the bulk phase of BESs and serum flasks. The biocathode samples were fixed by incubating in a fixative solution (2% glutaraldehyde and 2.5% paraformaldehyde) overnight at 4 °C. These were then dehydrated using different concentrations of ethanol (30%, 50%, 70%, 80%, 90%, and 100%) for 20 minutes each, which was followed by overnight dehydration in a silica desiccator. In the end, samples were coated with gold nanoparticles using JEOL JEC-1600 Auto-Fine Coater (JEOL Ltd., Japan) at 20 mA for 45 seconds and then were observed under a scanning electron microscope (JEOL JSM-6010 PLUS-LS, JEOL Ltd. Japan). An elementary analysis of the cathode surface was conducted by FEG-SEM-EDS (Hitachi SU8010 series, SAIF, Panjab University, Chandigarh).

16S rRNA amplicon sequencing-based microbial community analysis

Microbial biofilm grown at the cathode surface along with the sediment inoculum source were processed for community analysis. The genomic DNA was extracted by following the standard protocol according to the manufacturer's kit (DNeasy® PowerSoil® Pro kit Qiagen, Germany). It was then quantified using Nanodrop (Genova Nano – 4359, Jenway, Cole-Parmer, UK) and Qubit fluorimeter (V.3.0, Thermo Fischer Scientific, USA). The isolated DNA samples were used as a template to synthesize 16S rRNA, and the V3-V4 regions of 16S rRNA were amplified using a specific V3 forward primer 5′-GCCTACGGGNGGCWGCAG-3′ and V4 reverse primer 5′-ACTACHVGGGTATCTAATCC-3′. The amplicon sequencing was conducted by Eurofins Scientific, India, with Illumina MiSeq 2500 template. The relative sequence abundance of the enriched microorganisms was obtained by aligning the operational taxonomic units (OTUs) with the Silva

database at a 99% similarity level using QIIME. Later, the obtained results were visualized using R packages. The genera with a relative sequence abundance of lower than 1% are grouped as others. The OTU sequences used for visualization have been submitted to NCBI Sequence Read Archive (SRA) with NCBI-SRA: SRR12506991 accession number.

QUANTIFICATION AND STATISTICAL ANALYSIS

Mean and standard deviation was used to calculate the average and amount of dispersion in the respective experiments.